

Diphtheria Toxin: Mode of Action and Structure

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INTRODUCTION

The wealth of biochemical knowledge accumulated in recent years has permitted fruitful study of the molecular mechanisms of pathogenicity in a wide variety of diseases. In bacterial diseases many aspects of the host-parasite interaction still remain snarled in complexity, but certain factors have become amenable to study. Perhaps the most notable advances have occurred in diseases, such as diphtheria and cholera, in which a single, potent exotoxin is responsible for the major symptoms.

In diphtheria the existence of such a toxin has been known for almost a century, and its action has been studied in a variety of systems almost from the time of its discovery. Only after the basic framework of knowledge of protein synthesis was established in the 1950s, however, did such studies begin to yield a consistent pic-

ture. At the present time there is good reason to believe that the toxicity of the molecule is due to inhibition of protein synthesis in the human host, and certain aspects of the biochemistry of its action are known in detail.

OUTLINE OF THE PROBLEM

The causative organism of diphtheria, *Corynebacterium diphtheriae*, is normally found only in the upper respiratory tract of men, cattle, and horses. Infections in man may remain subclinical, or the bacillus may proliferate extensively upon and within the superficial epithelial layers of the pharynx, nasopharynx, or upper trachea. Such proliferation commonly results in the formation of a leather-like *pseudomembrane*, which is a characteristic diagnostic feature of the disease. Less commonly,

infections of wounds or the skin or mucous membranes occur at peripheral sites. However, regardless of the location of the primary infections, there is little invasion of underlying tissues, and the bacillus is rarely found in significant numbers in the blood or internal organs.

The symptoms, in contrast to the causative agent, are not localized (2). Death can result from suffocation caused by occlusion of the air passage by the pseudomembrane, but more often it is attributable to damage to internal organs, distant from the site of infection. In severe or fatal cases, tissue necrosis at gross or microscopic levels and various physiological changes are observed in many organs, including the heart, kidneys, liver, lungs, nervous system, and others. Cardiac failure is frequently cited as the immediate cause of death (28), but physiological alterations in other organs certainly contribute and may often be the determining factor.

Loeffler observed in 1884 that injections of experimental animals with the diphtheria bacillus produced localized infections with widespread tissue damage similar to that in the clinical disease (101). He suggested that the damage at distant sites might be caused by a toxic substance produced by the pathogen and transported throughout the body. In 1888 Roux and Yersin demonstrated that culture filtrates of the diphtheria bacillus caused death of experimental animals with a similar pattern of tissue damage (141). Some five decades later the toxic product was obtained in sufficiently pure form to ascertain that it was protein in nature (49, 120) and it is now clear that it is a single protein (33, 46, 47, 58, 160).

That the toxin is indeed of major importance in clinical diphtheria is shown not only by the similarity of the symptoms produced by purified toxin in experimental animals, but also by the fact that immunity to the toxin provides protection against severe symptoms of the disease. In recent years mass immunization against diphtheria toxoid (toxin detoxified by treatment with formaldehyde) has led to a remarkable decrease in the incidence of clinical diphtheria in many countries (24) and might result in eradication if a sufficiently high percentage of the population were immunized.

Despite the proven role of the toxin, toxigenicity in *C. diphtheriae* is not synonymous with pathogenicity (3, 5, 73). Nontoxic strains have been isolated, and certain of these not only survive for long periods in the upper respiratory tract, but also are able to produce mild to moderately severe infections (5, 50). Although

the more dramatic symptoms seen in severe infections by toxigenic strains are lacking, pseudomembrane formation does occur. Furthermore, merely the capacity to produce the toxin does not make a strain pathogenic. The PW-8 strain, which is used throughout the world to produce high titers of toxin for preparing toxoid or for research, appears to be relatively avirulent (97). The pathogenicity of *C. diphtheriae* is therefore complex. Toxin formation can dramatically increase the severity of an infection, but is neither necessary nor sufficient for survival or pathogenicity of the bacillus.

In 1951 Freeman made the remarkable discovery that toxigenicity in *C. diphtheriae* is correlated with infection by certain temperate bacteriophage (53). This observation was subsequently confirmed and extended by Barksdale, Groman, and others (4-7, 75-78). Lysogenization of a nontoxic strain with phage carrying the *tox*⁺ gene converted it to toxigenicity, and curing of phage infection yielded a nontoxic strain. The implication of these results that the structural gene for diphtheria toxin might reside on a phage genome now seems virtually beyond doubt (63, 81, 100, 115, 158). Toxin is synthesized in a cell-free system from *Escherichia coli* programmed with DNA from corynephage β , carrying the *tox*⁺ gene (106), and other work with mutants of the same phage also implies that the phage genome codes for the toxin (158). The toxin is therefore, by definition, a phage protein.

Diphtheria toxin has been purified, crystallized, and partially characterized in many laboratories. It is an acidic, globular protein ($pI \approx 4.1$) with a molecular weight most recently estimated at 62,000 to 63,000. As far as is known, it contains no unusual amino acids, no nonprotein moieties, and no other gross features to distinguish it from a wide variety of other nontoxic proteins (91, 134). It is not as potent on a weight or molar basis as certain other bacterial toxins, such as those of the botulinum and tetanus bacilli, but its toxicity is remarkable nonetheless. About 25 ng of the toxin injected subcutaneously into a 250-g guinea pig is sufficient to cause death in 4 to 5 days (thereby defining a standard minimum lethal dose [MLD]), and less than one-thousandth of this amount injected intradermally into the shaved back of a rabbit or guinea pig produces a visible necrotic reaction. Many animals, including man, monkeys, rabbits, and various fowl, are about as sensitive as the guinea pig on a body weight basis, and it may be calculated that a few micrograms is sufficient to cause death of an unimmunized adult human. However, with

rats and mice, doses about 3 orders of magnitude greater per unit body weight are required for comparable responses. Inasmuch as the toxin is unstable at acid pH, it has no effect when administered orally.

A guinea pig injected with a lethal dose of the toxin remains normal in appearance and behavior for several hours and then gradually begins to show signs of weakness and lethargy, which progress until the animal goes into shock and dies a few days later. Even when a massive amount of the toxin is administered (several thousand lethal doses), the animal shows no symptoms immediately and never dies before about 10 h. Thus, a period of several hours to days is required for the toxin's effects to be manifested. Its ultimate effect becomes irreversible much sooner, however. With a dose of 10 MLD, for example, even a large excess of antitoxin administered 1 h later will not prevent death. It is probable that the period of reversibility corresponds to the time required for absorption of a lethal dose by the cells.

The mechanism by which the toxin causes death has been studied extensively in whole animals. As indicated above, gross and microscopic tissue damage is found in a variety of internal organs (the heart, suprarenals, kidneys, liver, pancreas, diaphragm, nervous tissue, and others). The specific pattern varies somewhat from species to species, but in all there are widespread morphological changes (2). Similarly, and not surprisingly, multiple physiological changes occur. For example, toxin-treated animals show decreased levels of muscle phosphocreatine (129), reduced capacity to metabolize lactic acid (45) and to synthesize carbohydrates (40), increased resistance to insulin (38), reduced cardiac capacity (164), increased levels of potassium in the blood (164), and so forth. It is conceivable that such diverse effects might follow from a specific action of the toxin on a particular organ or cell type. However, it was long suspected that the toxin may be relatively nonspecific.

Support for this notion has come from studies in cell culture (see reference 146 for a review). The toxin has been shown to be lethal for primary and continuous cell cultures from a variety of animals (54, 99, 130). The first morphological changes in monolayer cultures of HeLa cells, for example, may be detected within several hours (3 h in one study; 106) after addition of high concentrations of toxin. Increased granularity is observed, followed by rounding up of the cells and release from the glass surface (Fig. 1). Lysis and disintegration

do not seem to occur for at least some hours after release, however (109, 146).

The relative sensitivities to toxin of cell cultures from humans and various animals seem to correspond approximately to those of the parent animals (54, 145). Thus cells from humans, monkeys, or chickens, for example, are killed by relatively low concentrations of toxin, whereas those from mice and rats require concentrations orders of magnitude higher for similar effects. Cultures from various organs within a given animal species are similar in sensitivity. Despite the complications of de-differentiation and selection in culture, morphologically distinct cell types, including embryonic heart, fibroblastic, and epithelial types show no obvious differences in sensitivity to the toxin (19, 54). This correlates with the apparent lack of strong tissue specificity observed *in vivo* and implies that the toxin acts on a process common to many if not all cell types.

INHIBITION OF PROTEIN SYNTHESIS

Studies in Cell Culture

The first indication of an effect of diphtheria toxin on protein synthesis was observed by Strauss and Hendee in 1959 (150). While studying the effect of the toxin on respiration in HeLa cells (no significant effect was found), they observed that accumulation of protein was reduced over the 12-h period of the experiment. Subsequently, they characterized the effect of toxin on the incorporation of [35 S]methionine into protein in these cells. After the toxin was added to a final concentration of about 10 nM, amino acid incorporation proceeded normally for 1 to 1.5 h and then rapidly came to a halt (Fig. 2). With lower toxin concentrations, the lag period was longer and the decline in the rate of protein synthesis more gradual, but higher concentrations failed to shorten the lag. Thus, about 10 nM toxin was saturating for this system. In another study slightly lower concentrations (3 to 5 nM) were required for saturation (109). (Concentrations of toxin are frequently expressed in terms of flocculating units [Lf] or MLD per unit volume. One Lf of toxin is equivalent to about 2.5 μ g of protein [8, 34]. The specific toxicity varies from preparation to preparation, but figures of 40 to 60 MLD/Lf are often reported.)

The presence of a lag seems to be invariable. However, there is a marked variation in the minimal duration, from as little as 15 min to at least 3 h, depending on the strain of cells and culture conditions employed (109, 123). The

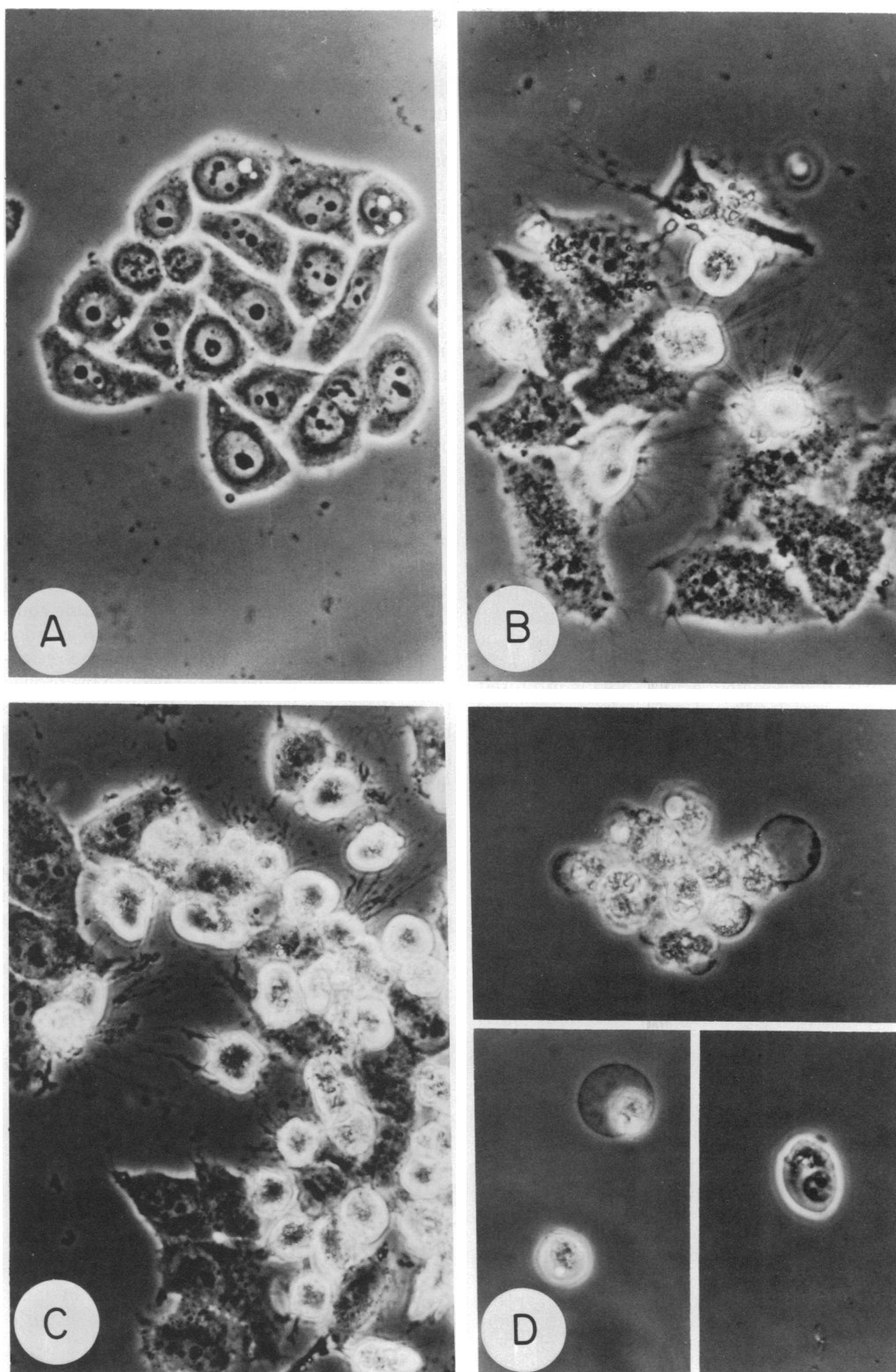


FIG. 1. Phase-contrast micrographs showing progressive intoxication of KB cells exposed to 24 nM toxin. Magnification: $\times 1,500$. (A) Normal cells; (B) exposed to toxin for 3 h; (C) exposed to toxin for 5 h; (D) exposed to toxin for 8 h. From reference 109 by permission of the author and publisher.

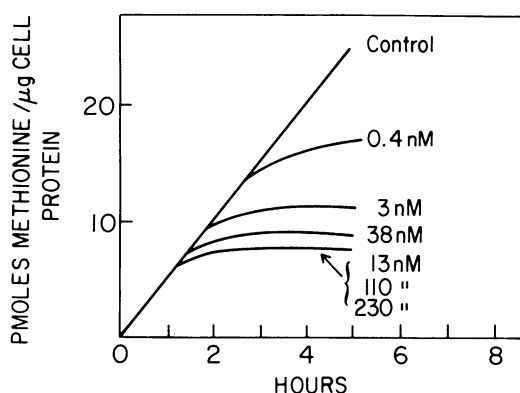


FIG. 2. Kinetics of incorporation of methionine into protein by HeLa cells after addition of diphtheria toxin. Radioactive methionine and toxin were both added at zero time. The concentration of toxin is indicated for each curve. Modified from reference 150 by permission of the author and publisher.

effect on protein synthesis can be prevented by adding excess antitoxin within a period which varies according to the toxin concentration.

Is the effect on protein synthesis direct or indirect? Synthesis of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) is inhibited in toxin-treated cells only after the cessation of protein synthesis (92, 149). Energy metabolism is also affected, but again apparently only secondarily. The rates of glycolysis and respiration and the concentrations of adenosine 5'-triphosphate (ATP), guanosine 5'-triphosphate (GTP), and hexose mono- and diphosphates all remain essentially unaffected for several hours after protein synthesis has ceased (34). An immediate effect of the toxin on phosphate incorporation into charcoal-adsorbable material (largely nucleotides) was reported (92), but this was not confirmed in subsequent studies (34, 123). Finally, there also appears to be no early effect on permeability of the plasma membrane, as evidence by the following: (i) the kinetics of transport of potassium ions into cells remains normal for at least 1 h after addition of toxin (92); (ii) leakage of phosphorus-containing compounds from $^{32}\text{P}_i$ -labeled cells is insignificant prior to the inhibition of protein synthesis (149); (iii) transport of amino acids occurs even after cessation of protein synthesis and the levels accumulated are equal to those of control cells (109, 122). In summary, the data available give no indication that the effect on protein synthesis might be secondary.

Studies in Cell-Free Systems

Cell-free systems of protein synthesis provided a means of testing for a direct effect of the

toxin on protein synthesis. Kato and co-workers showed inhibition in a cell-free system from guinea pig liver (90, 94), but the enormous concentrations of toxin required (0.5 mg/ml for half-maximal inhibition) gave reason to doubt the specificity of the effect. Subsequently Collier and Pappenheimer demonstrated a strong inhibition of protein synthesis (up to 95%) in crude extracts from HeLa cells by concentrations of toxin about 1,000-fold lower than those employed in the earlier work (Table 1) (35, 123). In addition, they found that extracts prepared from toxin-treated cells were about 80% less active in synthesizing protein than control extracts.

Further studies revealed that the inhibition by toxin was dependent on a soluble, low-molecular-weight component of cell extracts (35). This was noticed when a cell-free system from rabbit reticulocytes, prepared by different methods, was tested and found to be entirely insensitive to the toxin. Subsequently, it became apparent that the HeLa cell extracts still retained the low-molecular-weight soluble components from the parent cells, whereas such components had been removed from the reticulocyte fractions during preparation. This proved to be the critical difference; the HeLa extracts became insensitive to toxin after dialysis or passage through Sephadex G-25, and dialyzed HeLa extracts, or the reticulocyte system, could be resensitized by adding back the low-molecular-weight fraction from HeLa cells (boiled, ribosome-free supernatant fraction). The low sensitivity of the cell-free system prepared by Kato and co-workers (90) may have also been due to the absence of the essential dialyzable component, inasmuch as their preparative procedure would have been expected to remove such components.

The identity of the sensitizing factor was determined empirically (35). A variety of preparations of known cofactors were tested, and only

TABLE 1. Inhibition of leucine incorporation in undialyzed extracts from HeLa cells^a

Toxin concn (nM)	[^{14}C]leucine incorporated (counts/min)	Inhibition (%)
0	230	0
3	185	19
9	89	61
45	33	86
450	11	95
2,700	15	93

^a Modified from Table III of reference 35 by permission of the publisher.

those containing oxidized nicotinamide adenine dinucleotide (NAD^+) were found to sensitize dialyzed systems to the toxin. It was then shown that the factor chromatographed with NAD^+ on Dowex-1 ion-exchange resin, and that undialyzed HeLa extracts became insensitive to toxin after treatment with streptococcal NADase (Table 2). Goor and Pappenheimer later showed that the oxidized but not the reduced form of the cofactor was active (71).

Which component of protein synthesis is inhibited by toxin and NAD^+ ? Initial experiments showed that the transfer of labeled amino acids into polypeptide chains from preformed [^{14}C]aminoacyl transfer RNA (tRNA) was strongly inhibited, whereas the synthesis of aminoacyl-tRNA was not (29). Thus, the toxin-sensitive component was involved in the process of amino acid polymerization on the ribosome. Tests of the supernatant and ribosomal fractions then localized the effect to the former of these (29). Incubation of ribosomes or polyosomes with strongly inhibitory levels of toxin and NAD^+ had no effect on their integrity or activity, but the supernatant fraction was inactivated by a similar treatment.

At the time that these experiments were performed, the only components of the supernatant fraction known to be required for amino acid polymerization, besides GTP and certain inorganic ions, were two proteins now termed elongation factors 1 and 2 (EF-1 and EF-2; reference 27). (Earlier terms used for the same factors were transfer factors 1 and 2, and aminoacyl transferases 1 and 2.) EF-1 is required for attachment of aminoacyl-tRNA to ribosomes and EF-2 (molecular weight about 100,000; reference 37) is required for the translocation process. The latter process involves translocation of peptidyl-tRNA from the acceptor to the donor site on ribosomes and movement of mRNA by one nucleotide triplet after each round of peptide bond formation. GTP is required and is hydrolyzed to guanosine 5'-diphosphate (GDP) and inorganic orthophosphate during both processes.

As shown in Fig. 3, after treatment of reticulocyte supernatant fraction with toxin plus NAD^+ and separation of the elongation factors on Sephadex G-100, EF-2 shows a markedly reduced activity whereas EF-1 remains fully active (29). No effects on other components of the cell-free system were found, and it was concluded that EF-2 was probably the target of action of the toxin. From studies of extracts from toxin-treated HeLa cells, Goor and Pappenheimer also concluded that it was one of the elongation factors which was inactivated (70).

TABLE 2. *Effect of nicotinamide adenine nucleotidase (NADase) on the toxin sensitivity of undialyzed extracts from HeLa cells^a*

HeLa extract	[^{14}C]leucine incorporated (counts/min)		Inhibition (%)
	Control	Toxin (360 nM)	
Untreated	767	50	93
Pretreated with NADase	761	720	5
Pretreated with NADase ^b ; 250 μg NAD^+ added at time of initiation of incorporation reaction	802	320	60

^a Modified from Table IX of reference 35 by permission of the publisher.

^b Extracts were pretreated with 900 units of NADase per ml for 1 h at 0°C before addition to reaction mixtures.

In addition, their results suggested that the toxin-sensitive factor was accessible only when free in solution. A fraction of both elongation factors within cells is bound to ribosomes, in which form EF-2 is protected from inactivation by toxin and NAD^+ (59, 70, 144).

ADP-RIBOSYLATION OF EF-2

Identification of the Reaction

An important clue to the mechanism of inactivation of EF-2 came from the effect of nicotinamide, first observed by Goor, Pappenheimer, and Ames (72). Nicotinamide not only inhibited the inactivation, but at high concentrations (on the order of 30 mM) also promoted *reactivation* of the inactivated factor.

This finding and other data on the kinetics of inactivation were originally interpreted in terms of a model involving formation of a ternary complex between the toxin, EF-2, and NAD^+ . It was supposed that the complexed EF-2 was inactive and that nicotinamide dissociated the complex by competing with NAD^+ . The fact that a stoichiometrically equivalent amount of toxin would be required for inactivation of EF-2 detracted from the model, in view of estimates that only a few molecules of toxin might be sufficient to kill a cell. Later Gill et al. were unable to detect the predicted ternary complex (60). (There is evidence, however, that such a complex may exist under certain conditions [52].)

A more plausible, catalytic mechanism of inactivation of EF-2 was demonstrated by Honjo and co-workers in 1968 (83-85). A key experiment involved incubation of mixtures of toxin and purified EF-2 with each of a number

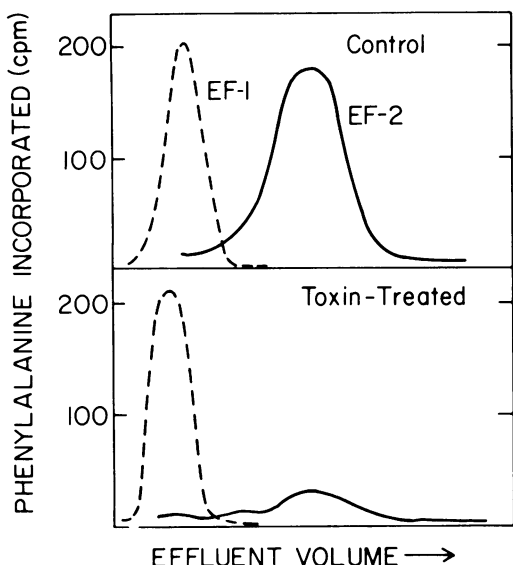
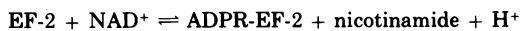


FIG. 3. Fractionation of normal and toxin-treated ribosome-free supernatant fraction from rabbit reticulocytes on Sephadex G-100. Upper frame, control supernatant fraction, incubated with NAD^+ in buffer before loading onto the column. Lower frame, supernatant fraction incubated with toxin and NAD^+ before chromatography. Modified from reference 29 by permission of the publisher.

of preparations of radioactive NAD^+ , labeled in various parts of the molecule. Stoichiometrically equivalent amounts of label were incorporated into the protein fraction (trichloroacetic acid-precipitable material) from all parts of NAD^+ except the nicotinamide moiety (Table 3). Label in nicotinamide was released free in solution. The protein-bound label was shown to be stably attached to EF-2; none was found associated with toxin in mixtures fractionated on hydroxylapatite. Also, the kinetics of attachment of label coincided with the time-course of inactivation of EF-2 (Fig. 4).

From these and other results, it was suggested that the toxin inactivated EF-2 by promoting attachment of the adenosine diphosphate ribose portion (ADPR) of NAD^+ (Fig. 5) (85):



Gill et al. independently obtained less direct evidence for this reaction (60), and subsequent work in many laboratories leaves no doubt that this mechanism explains the inactivation of EF-2. The reaction also provides a simple explanation for the reactivation by nicotinamide. Inasmuch as nicotinamide is a reaction product, high concentrations should shift the equilibrium position toward the left, thus reactivating EF-2.

Formally, the reaction should be termed $\text{NAD}^+:\text{EF-2}$ ADPR-transfer, but for simplicity I shall refer to it as ADP-ribosylation or ADPR-transfer. Certain important features of the reaction are discussed below. Other aspects will be considered in a later section.

ADPR is Covalently Attached

The chemical stability of the linkage between ADPR and EF-2 and the reversibility of the reaction imply that ADPR is covalently attached (85). The linkage resists treatment at 95 C for 6 min in 0.1 N HCl or 0.1 N NaOH, and is stable for longer periods in 5% trichloroacetic acid. Treatment with 1 N NaOH or 1 N HCl for similar periods causes partial hydrolysis, however. The reversibility of the reaction, as discussed below, implies that the ADPR-EF-2 linkage conserves a significant percentage of the bond energy of the original nicotinamide-ribose linkage of NAD^+ .

There is good evidence that EF-2 contains only a single attachment sight for ADPR (131, 140), but the nature of the linkage between ADPR and EF-2 is not yet fully characterized. ADPR must be linked through its nicotinamide mononucleotide (NMN) ribose moiety, inasmuch as ribose 5'-phosphate remains attached after removal of the adenosine 5'-monophosphate (AMP) portion with snake venom phosphodiesterase (82, 83). However, the side chain of EF-2 to which ADPR is attached remains uncertain. The amino acid sequence of a 15-residue tryptic peptide surrounding the residue has been determined as Phe-Asp-Val-His-Asp-Val-Thr-Leu-His-Ala-Asp-Ala-Ile-X-Arg, where X represents the attachment site (138, 139). Residue X is weakly basic and does not correspond to any amino acid commonly occurring in proteins. It is present even prior to contact with

TABLE 3. Incorporation of label from NAD^+ into acid-insoluble fraction^a

NAD ⁺ employed	Incorporation (pmol)
$\text{NAD}^+-(\text{adenine})-^{14}\text{C}$	51.2
$\text{NAD}^+-(\text{adenosine})-^3\text{H}$	50.6
$\text{NAD}^+-(\text{both phosphates})-^{32}\text{P}$	50.0
$\text{NAD}^+-(\text{ribose in NMN})-^{14}\text{C}$	50.0
$\text{NAD}^+-(\text{nicotinamide})-^{14}\text{C}$	0.3

^a Reaction mixtures contained tris(hydroxymethyl)aminomethane buffer, EF-2 from rat liver, and diphtheria toxin, in addition to the radioactive NAD^+ indicated. After incubation for 10 min at 37 C, the trichloroacetic acid-insoluble material was collected on filters and counted. From reference 84, by permission of the authors and publisher.

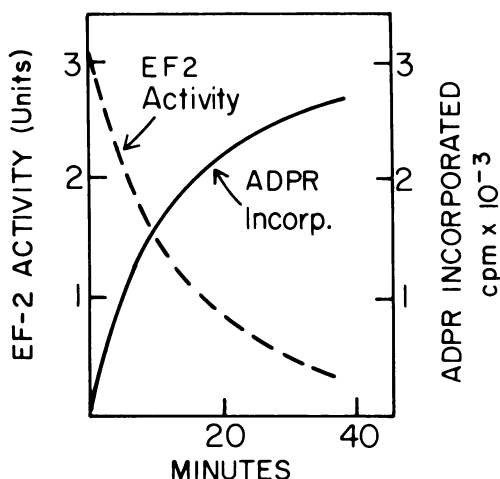


FIG. 4. Kinetics of inactivation of EF-2 and of incorporation of ADP-ribose into the acid-insoluble fraction. Redrawn from reference 84 by permission of the author and publisher.

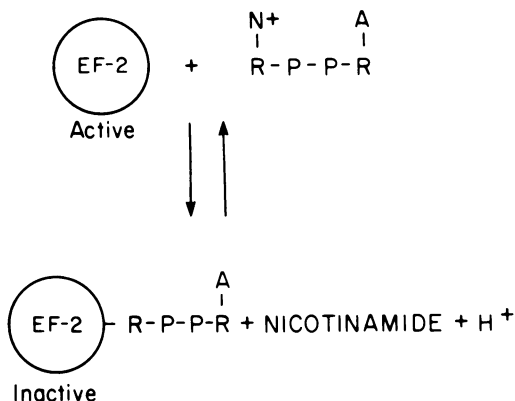


FIG. 5. ADP-ribosylation of EF-2.

toxin and thus may result from a modification of the primary translation product.

The Role of Toxin is Catalytic

Stoichiometric considerations imply a catalytic role for toxin. Thus, a molar ratio of ADPR-EF-2 to toxin of at least 50 is attainable (83). Also, the initial rate of the reaction is dependent on the concentrations of both toxin and EF-2, whereas the amount of product formed at completion depends only on the concentration of EF-2. From these facts alone one can not exclude the possibility that toxin acts as a cofactor rather than an enzyme (that is, that it may be covalently altered and cyclicly regenerated). Recent work has not supported this possibility, however (89).

Later work showed that it is not actually the whole toxin molecule but a *proteolytic fragment*

thereof (fragment A), which catalyzes the ADP-ribosylation of EF-2 (30, 33, 58). This fact and the activation process will be discussed below. For simplicity, I shall continue to refer to the enzymatic activity of toxin, implying the ADP-ribosylation activity of the active fragment.

Substrate Specificity

Specificity for both the protein and pyridine nucleotide substrates is high, but not absolute. Under normal reaction conditions EF-2 from all eukaryotic organisms so far tested (including vertebrate and invertebrate animals, wheat and yeast) is active as substrate, whereas other proteins are not (72, 79, 85, 124). For example, if one ADP-ribosylates a crude, high-speed supernatant fraction from rabbit reticulocytes in the presence of labeled NAD⁺ and analyzes the product on sodium dodecyl sulfate (SDS)-gels, the only detectable label is found in the EF-2 band (J. Traugh and R. J. Collier, unpublished results). However, at very high concentrations of toxin, or of the active fragment of toxin, other proteins may be ADP-ribosylated, including the toxin itself (55). This phenomenon is discussed below in relation to the mechanism of catalysis. If nuclei or nuclear fragments are present in the reaction mixture, other labeled proteins may be detected as a result of a competing reaction catalyzed by a nuclear enzyme (116, 151). This enzyme uses NAD⁺ as substrate in forming a polymer of ADPR which may be attached to protein. There appears to be no relation between this enzyme and toxin. Proteolytic degradation of ADPR-EF-2 also may produce labeled peptides of lower molecular weight than the unmodified factor. Labeled material of molecular weight about 40,000 has been found in aged [³H]ADPR-EF-2 (37).

It is interesting that EF-2 from rats and mice is apparently as active as substrate as that from more sensitive animals (72, 106). The simplest explanation for the resistance of rat and mouse cells to toxin would be a strong permeability barrier, perhaps due to the absence of specific receptors which may be present on more sensitive cells. It is also noteworthy that EF-G, the bacterial elongation factor corresponding in function to EF-2 and the analogous factor from mitochondria, are inactive as substrates (88, 137). There is evidence that toxin inhibits protein synthesis in bacterial cell-free systems under appropriate conditions, but NAD⁺ is not required and the mechanism appears to be entirely different (74, 156). Nothing is known about the significance of this action.

The substrate specificity of the pyridine nucleotide involved in ADP-ribosylation is also

high, and it is probable that only NAD^+ is utilized in vivo. Of other pyridine nucleotides tested only certain unnatural analogues of NAD^+ with minor structural changes can substitute (71, 85, 89). Thionicotinamide- AD^+ has a K_m (5 to 10 mM) similar to that of NAD^+ (8 mM), whereas acetylpyridine- AD^+ and deamino- NAD^+ have K_m values about 10-fold higher. Oxidized NAD phosphate (NADP^+), reduced NAD (NADH), reduced NADP (NADPH), and NMN are all inactive as substrates.

Reversibility and Thermodynamics

The equilibrium position of the reaction lies far to the right under normal conditions, but may be shifted toward the left by adding high concentrations of nicotinamide (72, 82, 85, 132), as indicated above. Honjo and co-workers demonstrated removal of attached ADPR and reactivation of EF-2 by incubation of ADPR-EF-2 with nicotinamide and toxin, and in addition have recovered an equivalent amount of authentic NAD^+ generated by the reversal (85). As predicted by the reaction equation, the equilibrium is further shifted toward the left (and the reverse reaction accelerated) by lowering the pH somewhat. The pH optimum of the reverse reaction is 5.3. Attempts to rescue toxin-treated cells with nicotinamide have failed (72, 105).

From the equilibrium position in the presence of various concentrations of nicotinamide the equilibrium constant has been determined (85):

$$K = \frac{(\text{ADPR-EF-2})(\text{nicotinamide})(\text{H}^+)}{(\text{EF-2})(\text{NAD}^+)} = 6.3 \times 10^{-4} \text{ M}$$

From this value one may calculate the standard free energy change (ΔF°) as -5.2 kcal per mol at pH 7 and 25 C (85). Inasmuch as the free energy of hydrolysis of the nicotinamide-ribose linkage of NAD^+ is known to be about -9.2 kcal per mol, the value for hydrolysis of the ADPR-EF-2 linkage must be about -4.0 kcal per mol at pH 7 and 25 C.

The major implication of these findings with respect to the reaction in vivo is that the inactivation of EF-2 at equilibrium should be virtually complete under physiological conditions. At pH 7 and a concentration of $10 \mu\text{M}$ NAD^+ (certainly an underestimate), less than 0.01% of the free EF-2 would be expected to remain in the unmodified, active form. This is consistent with the virtually complete inhibition of protein synthesis observed in tissue culture.

Factors Affecting the Rate of the Forward Reaction

Reversal of the reaction probably does not occur to a significant extent in vivo, and thus the rate of the forward reaction should primarily determine the rate of inhibition of protein synthesis. The forward reaction is affected by a number of variables, but uncertainties in many of these permit little confidence in calculations of the rate of inactivation of EF-2 in vivo at given concentrations of toxin or active toxin fragment.

The transfer of ADPR to EF-2 has a pH optimum of 8.2 to 8.5, but is rapid even at pH 7 (33, 69, 73, 84). In our experience there are no requirements for specific ions (33). A slight stimulation by Mg^{2+} has been reported (69), but we have found no inhibition by ethylenediaminetetraacetic acid (EDTA), except for that due to its contribution to ionic strength. The reaction is sensitive to ionic strength, being inhibited 25 to 40% by 30 mM NaCl , KCl , or NH_4Cl (33). MgCl_2 and magnesium acetate are each about 10-fold more inhibitory on a molar basis.

The finding that thiols stimulate the reaction (30, 36) appears to be due solely to their capacity to promote reductive activation of the toxin (33, 60) as described below. Thiols are also required for protection of EF-2 activity in protein synthesis (111), but *N*-ethyl-maleimide treatment does not block its activity as substrate for ADP-ribosylation (60). However, another sulfhydryl reagent, *p*-hydroxymercuribenzoate, does inactivate EF-2 as substrate (140), suggesting that certain critical sulfhydryls may be reactive with this but not the former reagent.

Certain compounds related to NAD^+ inhibit the reaction by competing for the NAD^+ binding site on the active toxin fragment. The most potent inhibitor of this type is adenine (74, 89), but its K_i ($40 \mu\text{M}$) is still well above the K_m of NAD^+ ($8 \mu\text{M}$). Adenosine is less effective than adenine by at least an order of magnitude, and AMP, ADP, ATP, ADPR, and NADP^+ are less inhibitory by two orders of magnitude (69, 89). Nicotinamide ($K_i \sim 0.2 \text{ mM}$) also inhibits the reaction weakly.

NADH has been found to inhibit weakly (K_i $30 \mu\text{M}$) if at all in assays with EF-2 from rabbit reticulocytes (89), although an early report suggested a strong inhibition (K_i $0.2 \mu\text{M}$) with rat liver EF-2 (69). The results in the reticulocyte system are supported by the finding that NADH binds only weakly to the active fragment of toxin (89). Inasmuch as the ratio of the

intracellular concentration of NAD^+ to its K_m is likely to be >1 , whereas the ratio for all the inhibitors listed is probably <1 , such inhibitors probably affect the reaction very little *in vivo*. Other, unnatural analogues of NAD^+ inhibit the reaction (89), but can be of no physiological significance.

The rate of ADP-ribosylation is also affected by interactions of EF-2. EF-2 binds to ribosomes (to the 60S subunit [154]) in the course of the translocation process and is apparently inaccessible to ADP-ribosylation while bound (59, 70, 96, 133). Gill and Dinius have estimated that over 75% of EF-2 is ribosome bound in extracts from rat liver (57). The rate of release of EF-2 during protein synthesis may be the limiting factor in the inactivation process under certain circumstances and may at least in part determine the duration of the lag period before protein synthesis is affected in toxin-treated cells (60).

EF-2 can also bind GTP, GDP, and various types of RNA free in solution (81, 113, 153, 155). EF-2 binds to ribosomes as a complex with GTP, and the interaction with RNA may reflect a functional interaction of the factor during translocation on ribosomes. At physiological levels of GTP or GDP, well above the apparent K_D values (10^{-6} to 10^{-7} M), we have found little inhibition of ADP-ribosylation (80, 113, 155), although there is a report that millimolar concentrations do inhibit (69). It is also unlikely that the interaction with RNA inhibits the reaction to a significant degree *in vivo*. Although the RNA-bound factor does seem to be protected from ADP-ribosylation, the interaction with RNA is strongly inhibited by physiological concentrations of GTP or GDP (Fig. 6) and is also sensitive to physiological ionic strengths (155).

In this context one may ask why EF-2 loses activity in protein synthesis when the ADPR group is attached. ADPR-EF-2 binds GTP normally (11, 113, 148) and the ADPR-EF-2:GTP complex binds to ribosomes (11, 12). The interaction with RNA appears to be blocked by the ADPR group, however (155). Nothing is known of the detailed molecular interactions of EF-2 during translocation, except that certain ribosomal proteins appear to be involved in binding EF-2 to the ribosome (157). However, from the fact that at least two types of RNA, messenger RNA (mRNA), and peptidyl-tRNA undergo changes in location on the ribosome during this process, it is not unlikely that there may be an essential RNA:EF-2 interaction (155). Thus, polypeptide chain elongation may

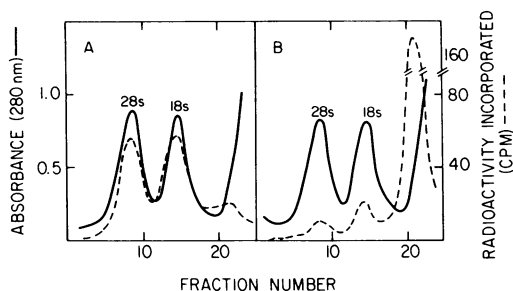


FIG. 6. Sucrose-density gradient centrifugation of mixtures of EF-2 and mammalian ribosomal RNA in the presence or absence of GTP. Direction of centrifugation, from right to left. Mixtures of rRNA and EF-2 (frame A) or ribosomal RNA, EF-2, and GTP (frame B) were layered on separate sucrose gradients and centrifuged. The gradient fractions were assayed for absorbance at 260 nm, and the EF-2 content was measured by reaction to completion with radioactive NAD^+ and a high concentration of diphtheria toxin. Redrawn from reference 153 by permission of the publisher.

be arrested at the pretranslocation step and with the ribosomes containing an ADPR-EF-2:GTP complex.

STRUCTURE-ACTIVITY RELATIONSHIPS IN THE TOXIN

Whole Toxin is a Proenzyme

Before the ADP-ribosylation activity was discovered, diphtheria toxin had been purified, crystallized, and partially characterized with respect to certain physical and chemical properties (136, 163), but its structure remained largely unstudied in detail. More recent work has revealed interesting structure-activity relationships. Most importantly, it has become clear that the ADP-ribosylation reaction is not catalyzed by whole toxin, but rather by a proteolytic fragment from the toxin (30, 33, 46, 56, 58, 104). Hence, the toxin itself is actually a proenzyme.

The toxin is apparently excreted from diphtheria bacilli as a single, intact polypeptide chain (molecular weight 62,000 to 63,000) containing two disulfide bridges and no free sulfhydryls. Although toxic for animals, this form is virtually, if not entirely, devoid of enzymatic activity. Such activity may be elicited by treatment with a protease, such as trypsin, followed by reduction of disulfide linkages. This treatment preferentially splits the molecule into two large fragments, A (molecular weight 24,000) and B (molecular weight 38,000 to 39,000). Fragment A is highly active in catalyzing ADP-

ribosylation of EF-2, and together with traces of other related fragments appears to account for all the enzymatic activity attributed earlier to whole toxin. Fragment B appears to have no enzymatic activity.

The cleavage and reduction processes may be demonstrated by electrophoresis on polyacrylamide gels in the presence of SDS (Fig. 7). A portion of the pure toxin (20% in this preparation) is commonly in the nicked form before exposure to trypsin, due to the action of bacterial proteases. This "naturally-nicked" toxin dissociates into fragments A and B which are indistinguishable (at least at a gross level) from those produced by trypsin nicking.

The activation process is shown diagrammatically in Fig. 8, together with certain properties of the reaction products. Primary structure studies have shown that the active A fragment corresponds to the N-terminal portion of intact toxin (31, 104). The proteolytic specificity required to generate the fragment needs further study, but trypsin, which cleaves specifically at basic amino acid residues, is highly effective whereas chymotrypsin is inactive (46). Pronase is also active. Within the nicked toxin, frag-

ments A and B are linked by a single disulfide bridge, and fragment B contains a second bridge internally within 17,000 daltons of the C-terminal end (46, 60).

Fragments A and B tend to remain associated through noncovalent forces after reduction of the nicked molecule (58, 160). It is clear that fragment A is enzymatically active in the free form, but it is uncertain whether or not dissociation from B is absolutely necessary for activity. It is possible that NAD^+ , EF-2, or both, may promote dissociation, but this has not yet been studied.

The activity of toxin detected in cell-free systems of protein synthesis before the activation process was discovered was dependent on a fortunate combination of circumstances; namely, the facts (i) that the naturally nicked form is present in at least small amounts in essentially all preparations of toxin, and (ii) thiols are routinely included in cell-free, protein-synthesizing systems. Ironically, the thiols are added largely to *preserve* the activity of EF-2 (111). In their presence the nicked form of toxin is reduced, permitting active fragment to be released. Free fragment A is commonly

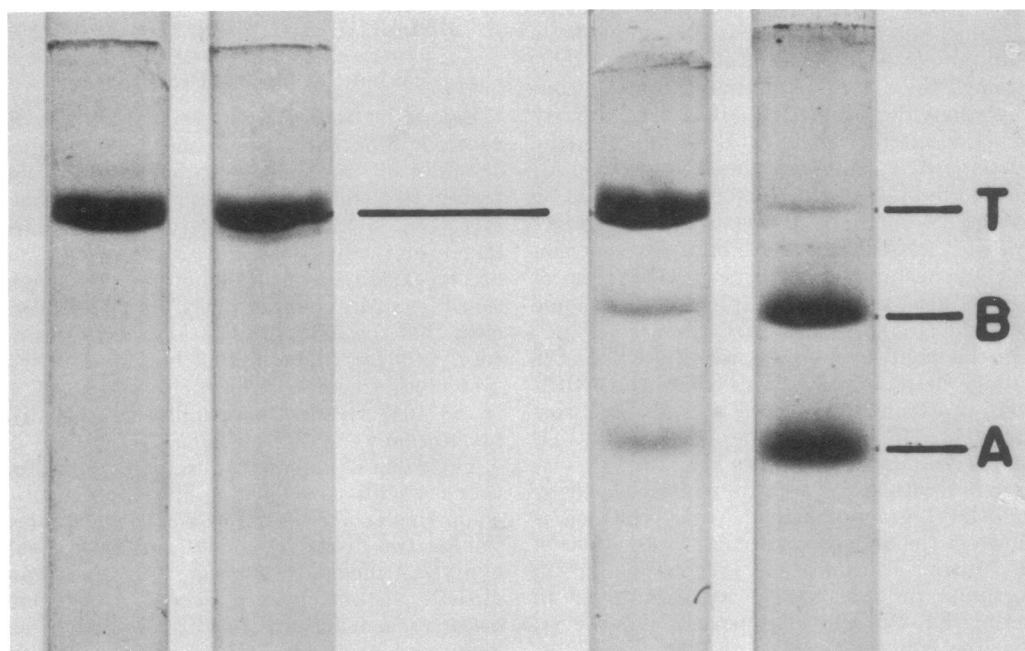


FIG. 7. Electrophoresis of trypsin-treated and untreated toxin on SDS gels. Identical samples of toxin were incubated in the presence or absence of $1 \mu\text{g}$ per ml of trypsin for 45 min. Duplicate portions of each sample were then treated at 100°C in 1% SDS either with or without mercaptoethanol, and a portion of each was subjected to electrophoresis on an SDS gel. The gels (from left to right) show samples treated, without trypsin or thiol, with trypsin only, with thiol only, and with both trypsin and thiol. The positions of fragments A and B and of intact toxin are indicated in the figure. Reproduced from reference 46 by permission of the publisher.

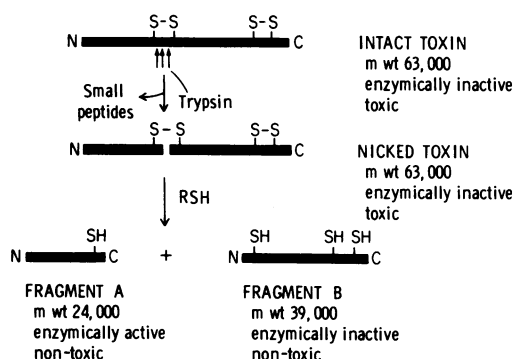


FIG. 8. Sequence of events in the expression of enzymatic activity (ADP-ribosylation of EF-2) in diphtheria toxin.

present in impure toxin preparations and may have been another source of activity in some experiments (30).

Toxicity of the Various Fragments and Forms

Preparations of toxin entirely free of the nicked form are not available, but samples originally containing 80% intact toxin appear to have the same toxicity for guinea pigs either before or after nicking with trypsin (46, 58). (Lethality tests used for these estimates are usually not more accurate than $\pm 20\%$.) Similarly, measurements of the kinetics of inhibition of protein synthesis in HeLa cells reveal only small differences between nicked and 80% intact samples. With the fully nicked material the lag period is reduced slightly, but the kinetics are otherwise similar (46). This suggests that HeLa cells contain a mechanism for nicking intact toxin, and that this step is not rate limiting.

Fragment A, despite its enzymatic activity, is not toxic for guinea pigs in doses of 8 nmol per kg body weight (46, 58). In contrast, whole toxin is lethal at molar concentrations 2,000 times lower (4 pmol per kg body weight). It has also been shown that fragment A does not inhibit protein synthesis in HeLa cells at concentrations up to 4 μ M. These somewhat surprising results suggest that fragment B is also required for toxicity. Work with nontoxic, immunologically cross-reacting forms of toxin has confirmed this notion, and there is now good reason to believe that fragment B is needed for attachment of toxin to cells. Thus, free fragment A is inactive on whole cells probably because it cannot traverse the plasma membrane to a significant extent.

Tests of purified fragment B have revealed no

toxicity or ADP-ribosylation activity. Although the instability of the fragment (see below) makes such measurements suspect, the absence of these activities is supported by studies with certain nontoxic, mutant forms of toxin, described below.

Properties of Fragment A

A prominent feature of fragment A is its stability. It may be heated at 100 C at neutral pH or exposed to pH values of 2 or 12 at room temperature for brief periods with little loss of activity (46, 58). Also, it is relatively resistant to trypsin or chymotrypsin, especially in the presence of NAD^+ (31, 32, 89). On the other hand, fragment B denatures and precipitates rapidly after dissociation of nicked toxin and can only be maintained in solution with high concentrations of urea or guanidine, or with detergents. For these reasons, and because of its enzymatic activity, fragment A has been studied more extensively than B.

Figure 9 shows a tentative, partial amino acid sequence of fragment A based on work nearing completion at the University of California at Los Angeles (R. J. DeLange, R. Drazin, and R. J. Collier, unpublished data). The N-terminal sequence is identical with that reported earlier by Michel et al. for fragment A and whole toxin (104).

The most noteworthy aspect of the sequence as it stands is the heterogeneity at the C-terminus. From digests with CNBr it is possible to isolate three C-terminal peptides, which terminate in the sequences ---Asn-Arg-COOH, ---Asn-Arg-Val-Arg-COOH, and ---Asn-Arg-Val-Arg-Arg-COOH. Trypsin apparently attacks at any of three, closely spaced arginines, producing three major forms of fragment A. Each additional arginine adds an extra positive charge at neutral pH, which probably explains the electrophoretic heterogeneity of the fragment. Under nondenaturing conditions, the fragment forms three major bands on polyacrylamide gels, all of which are active (see Fig. 11) (89). It also separates into three bands on isoelectric focusing (pI values of 4.56, 4.72, and 4.85).

A second point to note in the sequence is that the single half-cystine residue is near the C-terminus (the fourth residue from the proximal arginine) (31). This fact, together with other evidence placing the corresponding half-cystine of fragment B near its N-terminus, indicates that the trypsin-sensitive region of the toxin is enclosed within a disulfide loop of limited size (it is no greater than 40 residues, but may be much smaller).

Reduction and dissociation of most preparations yield predominantly fragments A and B but other fragments are sometimes seen (Fig 10). Gill and Dinius have found certain preparations which contain a species of nicked toxin which yields fragments E and F (molecular weight 34,000 and 28,000, respectively) (60). Fragments E and F each contain an intact disulfide bridge and are apparently normally bound together only by noncovalent forces. Fragment F is enzymatically active and has the stability properties of fragment A, which it contains. How these unusual fragments are generated has not been studied but it may

involve a protease of different specificity from that producing A-B nicked toxin.

Although not consistently observed in other laboratories, it has been reported that the site of preferential cleavage by trypsin is altered depending on the presence or absence of thiols (60). Highly specific cleavage at the A-B junction was observed only in the presence of a thiol. In its absence, preferential cleavage occurred at a point 7,000 daltons from the N-terminus, producing fragment C (molecular weight 55,000) plus the N-terminal fragment, which was not recovered (Fig. 10). Fragment C was susceptible to further attack by trypsin at the A-B junction, producing fragments D (molecular weight 17,000) and B in disulfide linkage. Free fragment D obtained after reduction is not enzymatically active, which has led to speculation that A may be the smallest enzymatically active fragment. The fact that the variation in specificity of the site of cleavage by trypsin with thiols is not uniformly observed may be due to undefined differences among various toxin preparations.

A dimeric form of whole toxin (6.6 to 6.8S) has been found and constitutes a sizable fraction (sometimes 100%) of certain toxin preparations (67, 122). SDS causes dissociation in the absence of thiols (56). (However, Goor has reported that 0.33 M dithiothreitol in the presence of 0.08 M EDTA dissociates the dimer [67].) The dimeric form appears to have similar but perhaps slightly reduced toxicity compared to the monomer (67, 135). There is evidence that it may be formed during ammonium sulfate precipitation of the toxin (135).

There are numerous suggestions in the litera-

ture that other variations may exist in whole toxin, besides those which have been described. This is evidenced, for example, by fractional immunological reactivity with antiserum A (127), variations in pyridine nucleotide binding (103), and heterogeneous electrophoretic patterns (J. Kandel and R. J. Collier, unpublished data). Studies are under way to analyze and correlate some of these variations.

Free fragment A is often found in toxin preparations, and, in fact, discovery of this peptide in crude toxin was the first indication of an interesting structure-activity relationship in the toxin (30). The free fragment probably arises by reduction or disulfide interchange of naturally nicked toxin, promoted by low levels of thiols in the culture medium. The fact that the contaminating fragment does not readily form disulfide-linked dimers implies that its sulfhydryl group may have been oxidized or is in the form of a mixed disulfide, perhaps with a half-cystine from cystine in the culture medium. Free fragment B is not found in crude toxin, presumably because of its insolubility and susceptibility to proteolysis.

Nontoxic, Cross-Reacting Forms of Toxin

Various nontoxic or partially toxic, immunologically cross-reacting forms of toxin (CRMs) have been isolated recently (125, 158-162). Mutants of phage have been selected which induce the formation of such CRMs when they lysogenize nontoxigenic strains of *C. diphtheriae*. Analysis of the properties of these proteins has demonstrated the feasibility of the genetic approach to the study of the toxin and has yielded considerable information about its structure and activity.

Certain of the CRMs are the same size as intact toxin and presumably represent missense mutations (CRMs 197, 228, and 176), whereas two others (CRMs 30 and 45) are N-terminal fragments, perhaps resulting from chain-termination mutations (Table 4). In all five CRMs, the A-B junction remains susceptible to tryptic attack, thus permitting analysis of the peptide products on SDS gels. CRMs 30 and 45 are both enzymatically active and yield fragment A with the normal level of activity. Their lack of toxicity is due to the absence of large C-terminal sections of fragment B, thus underscoring the necessity of this portion for toxicity. CRM 45 contains only two half-cystine residues, which localizes the second disulfide within the missing 17,000- to 18,000-dalton, C-terminal peptide.

The fragment A portions of the other three CRMs all have altered activities. Fragment A

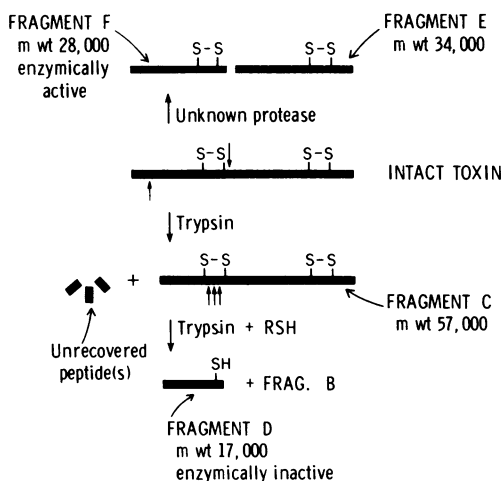


FIG. 10. Alternative modes of cleavage of intact toxin.

TABLE 4. *Some properties of diphtheria toxin and related proteins^a*

Protein	Mol wt	Toxicity (% of toxin)	Enzymatic activity		Receptor- blocking activity	Sizes of major tryptic products	Presumed structure
			Intact	Nicked			
Toxin	63,000	100	No	Yes		24,000 + 39,000	
Toxoid	63,000	0	No ^a	No ^a	None		
Fragment A	24,000	0	100		None		
CRM 30	30,000	0	Partial	Yes	Not done	24,000 + <10,000	
CRM 45	45,000	0	Partial	Yes	None	24,000 + 21,000	
CRM 176	63,000	<0.4	No	10%		24,000 + 39,000	
CRM 197	63,000	0	No	No	Yes	24,000 + 39,000	
CRM 228	63,000	0	No	No	10-15%	24,000 + 39,000	

^a From references 61 and 125 by permission of the authors and publishers (copyright 1973 by the American Association for the Advancement of Science).

from CRMs 197 and 228 is entirely inactive, which accounts for the fact that these CRMs are nontoxic. CRM 176, on the other hand, shows low but significant toxicity (0.2 to 0.4% of normal), and its fragment A is partially active (8 to 10% of normal) as described above.

Uchida and co-workers have elegantly demonstrated reconstruction of fully toxic molecules from complementary nontoxic CRMs containing defects in the A or B region (160, 161). In these experiments CRM 45, containing an active fragment A moiety, was treated with trypsin and dithiothreitol and mixed with another CRM similarly treated. The mixture was then dialyzed to remove the thiol and permit reoxidation. With CRM 197, as the complementary protein, toxicity was recovered after dialysis, and the toxicity per microgram of cross-reacting material was proportional to the ratio of CRM 45 to CRM 197. This indicates that B 197 was randomly associated with A 197 or A 45, thus implying complete exchange of the non-covalently-linked fragments. The formation of authentic disulfide-linked, 62,000-dalton hybrids under these conditions was demonstrated by incubating ¹²⁵I-labeled CRM 45 together with unlabeled CRM 197 and isolating the hybrid by diethylaminoethyl (DEAE)-cellulose chromatography.

When a high ratio of CRM 45 to complementary CRM is used (24:1), the hybridization test gives a good measure of the activity of fragment

B in the complement. With CRM 197 or CRM 176 the toxicity of the hybrid product is as high as that of nicked toxin, implying a normal fragment B in these proteins. Other tests, detailed below, confirm this prediction. With CRM 228, however, the hybrid has only 15% the normal level of toxicity, suggesting a defect in the fragment B portion. Again this result is supported by independent tests. It may be that CRM 228 is the result of a double mutation, one in region A and the other in B, but there may be other reasons for the partial activity of this CRM.

Mechanism of the ADP-Ribosylation Reaction

Our knowledge of the detailed mechanism of the ADP-ribosylation of EF-2 is based partly on a second, related reaction catalyzed by fragment A, namely the hydrolysis of the nicotinamide-ribose linkage of NAD⁺ (NAD⁺ glycohydrolase) (31, 89). Figure 11 shows that all three electrophoretic forms of fragment A have this activity, thus demonstrating that a contaminating NAD⁺-glycohydrolase is not responsible. The reaction occurs several orders of magnitude more slowly than the ADP-ribosylation of EF-2 and almost certainly does not contribute to toxicity. However, its existence implies a direct interaction of NAD⁺ with fragment A during the ADP-ribosylation of EF-2.

The interaction of NAD⁺ with fragment A has

been studied by dialysis and optical methods (31, 32, 89). Although the NAD^+ -glycohydrolase activity of the fragment makes equilibrium dialysis unsuitable, a dynamic dialysis method has been used successfully to show that fragment A contains a single NAD^+ binding site with a K_D of about $8 \mu\text{M}$ (Fig. 12). Bound NAD^+ quenches the fluorescence of tryptophan in fragment A, and a similar K_D is found by this method. This figure also correlates with the

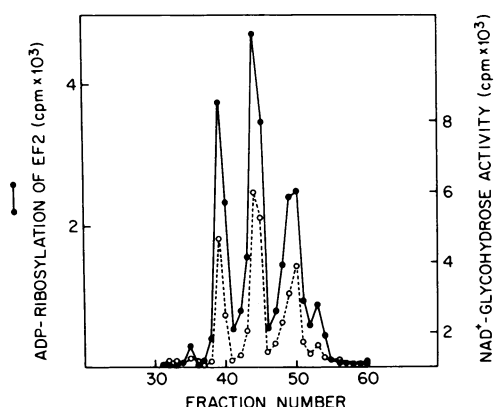


FIG. 11. NAD^+ -glycohydrolase and $\text{NAD}^+:\text{EF-2}$ -ADPR-transferase activities of fragment A eluted from nondenaturing polyacrylamide gels. A sample of fragment A was subjected to electrophoresis, and the gel was sliced into 2-mm thick fractions, each of which was eluted overnight in 0.1 ml buffer. The NADase and ADP-ribosylation activities were measured in each eluate. Fractions 1 to 29 and 61 to 90 showed background activities in both assays. Reproduced from reference 89 by permission of the publisher.

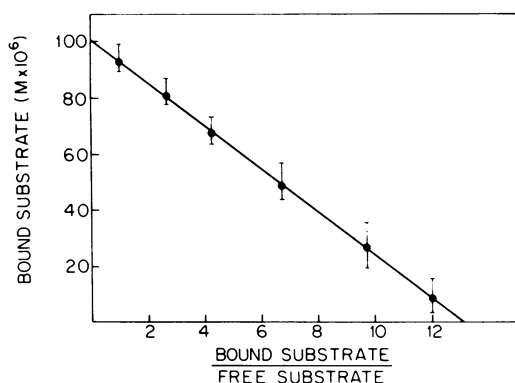


FIG. 12. Scatchard plot of data from dynamic dialysis measurements of NAD^+ binding to fragment A. From the y-axis intercept, there are 1.01 binding sites per 24,000 daltons of protein, and from the slope, $K_D = 8.3 \mu\text{M}$. The bars indicate the range of values obtained in five experiments. Reproduced from reference 89 by permission of the publisher.

value of K_M ($5 \mu\text{M}$) reported for NAD^+ in the ADP-ribosylation of EF-2 (69, 85).

Competition studies indicate that both the adenine and nicotinamide moieties of NAD^+ contribute to the binding to fragment A (89). Adenine and nicotinamide bind competitively with NAD^+ with K_i values of $30 \mu\text{M}$ and $200 \mu\text{M}$, respectively. Adenosine, adenosine phosphates, ADPR, and NMN bind at least an order of magnitude less strongly. Binding of NAD^+ , NMN, or N-methyl nicotinamide, all of which have charged pyridine nitrogens, results in quenching of fluorescence and induction of a weak, broad absorbance band with λ_{max} about 360 nm and ϵ about 500. These phenomena may result from a charge transfer complex between the charged nicotinamide ring and an indole ring of one of the three tryptophans in fragment A (89). This interaction would probably explain also the strong quenching of tryptophan fluorescence by NAD^+ .

The available evidence indicates that the interaction between NAD^+ and fragment A involves only noncovalent forces (69, 85, 89). Indication of a covalent ADPR-fragment A intermediate has been sought, but the results have been negative. Thus, for example, labeled nicotinamide does not exchange into NAD^+ in the presence of fragment A, and upon fractionation of mixtures of fragment A and adenosine-labeled NAD^+ no label is bound to the protein.

These results imply that the ADP-ribosylation of EF-2 proceeds through a ternary intermediate, containing fragment A, NAD^+ , and EF-2 (Fig. 13). The existence of NAD^+ -glycohydrolase activity in fragment A indicates that the nicotinamide-ribose linkage is labilized in the bound NAD^+ . EF-2 presumably binds to a site on fragment A adjacent to that of NAD^+ , and attachment of ADPR occurs by nucleophilic attack by the juxtaposed acceptor side chain of EF-2. The complex then dissociates into the products and fragment A.

This model is supported by data from kinetic studies (68, 69). These data also imply that interactions of the two substrates with fragment A must be independent; that is, each binds normally in the absence of the other. This has been demonstrated directly for NAD^+ and is being studied with purified EF-2. The nicotinamide-ribose linkage of NAD^+ bound to fragment A would also be attacked by water, but this is so slow under normal circumstances as to be entirely negligible.

It has been shown recently (C. Goff, personal communication) that other proteins beside EF-2 can be ADP-ribosylated by fragment A under unusual conditions. When bovine serum

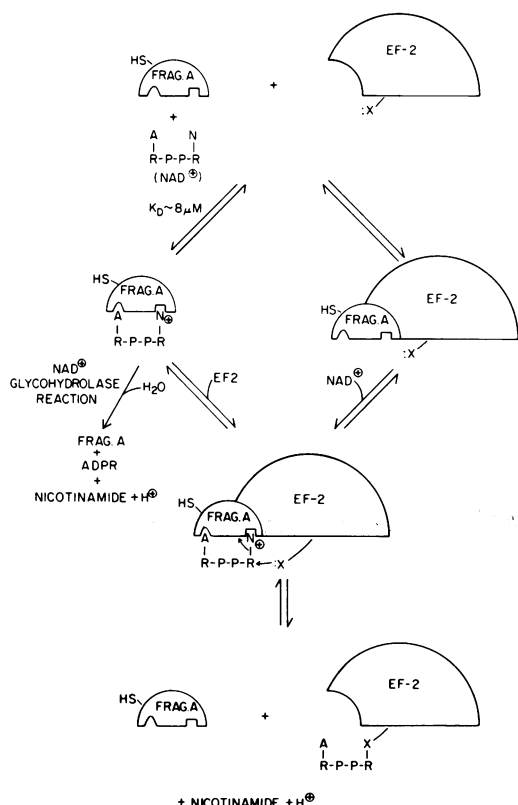


FIG. 13. Proposed mechanism for the ADP-ribosylation of EF-2 and NAD⁺-glycohydrolase activities of fragment A.

albumin or RNA polymerase core enzyme was incubated with adenine-labeled NAD⁺ and an extraordinarily high concentration of fragment A (20 mM, which is about 10^6 times greater than that normally employed in our assays), label was incorporated into the serum albumin, the β and β' subunits of RNA polymerase, and fragment A itself. The α subunit incorporated no label, and in those chains which were labeled the efficiency of labeling was low, with only about one ADPR incorporated per thousand molecules of protein. These results suggest that a variety of proteins may be reactive if the reaction is "forced," but EF-2 is probably the most efficient substrate by several orders of magnitude.

Pyridine Nucleotide Binding and ADP-Ribosylation Activity by Whole Toxin

When fragment A is linked to B within intact or nicked toxin it seems to be virtually if not completely inactive in ADP-ribosylating EF-2. Whether this lack of activity is due to interfer-

ence with the binding of either substrate, or both, or to other alterations of fragment A is not known. However, there is some pertinent information available.

Curiously, despite the fact that whole toxin cannot ADP-ribosylate EF-2, it seems to ADP-ribosylate itself if incubated long enough with NAD⁺ (55, 62, 64). The reaction requires hours to days to reach completion, and the maximal extent of labeling corresponds to only about 0.6 ADPR per toxin molecule. The intact form of toxin appears to be modified at about the same rate as the nicked form. Reduction is not required. There seems to be more than one site within toxin which may accept ADPR as evidence by the fact that incorporated label is found in both A and B fragments separated on gels, and also by the fact that the release of ADPR in 0.1 N NaOH does not follow first-order kinetics.

The available data suggest but do not prove that the ADP-ribosylation of whole toxin may represent an intramolecular modification; that is, one in which a given toxin molecule catalyzes attachment of ADPR to itself and no other molecules. Studies of the kinetics of the reaction as a function of the toxin concentration will be useful in deciding if this is indeed the case.

The existence of this reaction provides strong evidence that at least a fraction of the population of whole toxin molecules is capable of binding NAD⁺. The nature of the reaction suggests that the binding would be to the site on the fragment A moiety, and this is supported by the finding that CRM 197 lacks the self-modification reaction, as well as ADP-ribosylation and NAD⁺-glycohydrolase activities.

The binding of NAD⁺ and other pyridine nucleotides by whole toxin has been studied, but the problem needs still more work (103, 112, 147, 148). Measurements of quenching of toxin fluorescence by NAD⁺ (103) seem to indicate that the nucleotide binds to nicked with an affinity of the same order (K_D 9.7 to 13.6 μM) as that seen with fragment A, and to intact toxin with a slightly lower affinity (K_D 28 μM). However, the number of sites remains in doubt because of the relatively low quenching observed (which limits the accuracy of the data) and also because the calculated dissociation constants are about an order of magnitude greater than the concentration of toxin used. Michel and Dirx (103) have calculated that intact toxin binds two molecules of NAD⁺ whereas the nicked form binds only one. In contrast, preliminary experiments in my laboratory with dialysis techniques suggest that whole toxin binds far less than a molar equivalent of NAD⁺ regardless of whether it is nicked

or largely intact (J. Kandel and R. J. Collier, unpublished data). Unknown sources of variation among different toxin preparations may be responsible for these discrepancies. GTP has been reported to inhibit competitively the toxin NAD^+ interaction (148).

Two laboratories have reported that whole toxin binds NADH more strongly (K_D 0.5–0.7 μM) than NAD^+ (102, 103, 112). One batch of toxin which had been nicked by bacterial proteases seemed to contain two NADH binding sites, whereas another manifested only one site, either before or after nicking with trypsin (103). NADP and NADPH bound with affinities similar to NAD^+ and NADH, respectively, but there was not an exact correlation in the calculated number of binding sites. Although it is tempting to construct models on the basis of fluorescence data alone, it would perhaps be safe to await confirmation by results from dialysis experiments.

The question of whether whole toxin can bind EF-2 has not yet been studied carefully. There is a report that toxin forms a stable, ternary complex with NAD^+ and EF-2 (52), but another laboratory has sought such a complex without success, using different techniques (60).

IS THE ADP-RIBOSYLATION REACTION RESPONSIBLE FOR TOXICITY?

In cell-free systems there is little doubt that the ADP-ribosylation of EF-2 is responsible for the observed inhibition of protein synthesis, but one must question whether this reaction actually occurs *in vivo* and whether it is responsible for toxicity. Although there is still room for doubt, a strong case can be made for an affirmative answer.

(i) Persuasive evidence comes from a consideration of the properties of CRMs 176 and 197 (159). Each of these apparently contains a single amino acid substitution which alters the enzymatic activity of the molecules, and most importantly, it is found that *toxicity is affected similarly*. Thus, CRM 197 is devoid of enzymatic activity and is entirely nontoxic, whereas CRM 176 has a low specific activity in the ADP-ribosylation reaction and is partially toxic. The correlation is also valid for the inhibition of protein synthesis in tissue culture; CRM 197 is totally inactive and CRM 176 is partially active. The toxicity of CRM 176 (0.2 to 0.4% of normal) is not precisely the same as its specific activity (8 to 10% of normal), but this may be due to an altered stability or other factors operative *in vivo*.

Although one would like to have a larger number of mutants with specific alterations in ADP-ribosylation activity, the evidence provided by these two is strong. Unless toxin has a second, more important activity which is similarly affected by these mutations, toxicity must involve ADP-ribosylation. The two amino acid substitutions in question lie within the enzymatically active region of the peptide chain and do not significantly affect the function of the remainder of the toxin molecule.

(ii) There is good, although indirect, evidence that the ADP-ribosylation of EF-2 actually occurs in cultured cells treated with toxin. The definitive reaction product, ADPR-EF-2, has not been isolated from toxin-treated cells, but its existence is implied by the fact that protein synthetic activity of extracts from such cells is restored by nicotinamide (60, 105). (Additional toxin may be required to accelerate reversal of the ADP-ribosylation reaction [60].) Also, the total elongation factor activity (EF-1 and EF-2 combined) declines by 90% within the lag period of toxin-treated HeLa cells (60).

(iii) For reasons of complexity, the least compelling evidence comes from studies in whole animals. However, it appears that the data are generally consistent with the results from simpler systems.

Effects of toxin on protein synthesis in animals have been documented by two groups, but the pattern of inhibition seems to vary according to the site of injection, the dose, and perhaps other factors. Baseman et al. injected guinea pigs with 5 MLD toxin intravenously and measured protein synthesis in tissue slices prepared from animals sacrificed at intervals up to the time of death (30 h) (8). A decline was found in all organs tested, although in some the decline was preceded by a lag of several hours (Fig. 14). Bonventre and co-workers noted similar, widespread effects under certain conditions (20), but found preferential effects on musculature (cardiac, skeletal, diaphragmatic) under others (18, 20–22, 142). It may be that these results can be explained by a greater inherent sensitivity of musculature to toxin, which is manifested only at low toxin concentrations.

In another study, Bonventre reported little or no inhibition of protein synthesis in most tissues of guinea pigs injected with 1.4 MLD of toxin, if this total was divided into four equal doses which were administered at daily intervals (17). However, inhibitions ranging up to about 50% were observed in two tissues, pancreas and skeletal muscle. Such results may be variously interpreted for or against the assumption that the inhibition of protein synthesis explains toxicity. Unfortunately, we lack certain data

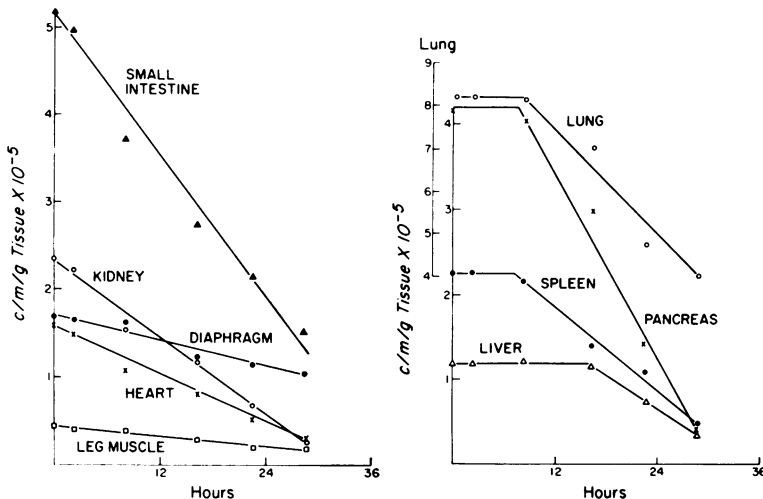


FIG. 14. Protein synthesis in tissue slices from intoxicated guinea pigs. Each animal (350 ± 20 g) received 5 MLD of toxin intravenously. At intervals thereafter, organs from the perfused animals were removed and tissue slices were prepared and incubated in the presence of [^{14}C]leucine. Perchloric acid-insoluble counts were determined and are indicated per gram wet weight of tissue. Reproduced from reference 8 by permission of the author and publisher.

necessary for a meaningful interpretation, such as the degree of inhibition required for lethality, and the degree of inhibition which occurs in various cell types composing the tissues studied. Such studies underscore the difficulties in studying pharmacological effects *in vivo*.

Unfortunately, no attempt has been made to isolate ADPR-EF-2 from toxin-treated animals. Measurements of EF-2 by Baseman et al. seemed to indicate a degree of inactivation proportional to the decline in protein synthesis, but no other enzymes were assayed as controls to demonstrate specificity (8). Other data suggesting reactivation of protein synthesis by nicotinamide in tissue extracts from toxin-treated animals are not entirely convincing (22).

Thus, the studies seem generally to show that inhibition of protein synthesis occurs in toxin-treated animals, but specificity remains to be demonstrated. Owing to the lack of precise data and the variables which apparently affect the pattern of tissue damage, no attempt will be made to analyze the secondary physiological changes or the ultimate cause of death in toxin-treated animals.

EVENTS PRECEDING THE ADP-RIBOSYLATION REACTION IN CELLS

To inhibit protein synthesis, the fragment A portion of toxin presumably must reach the cytoplasm. What is the mechanism of entry,

and what events occur subsequent to entry? Although our overall knowledge concerning these questions is meager, some pertinent data are available.

Attachment to Specific Receptors

The fact that fragment A and CRMs 30 and 45 do not inhibit protein synthesis in intact cells, even though they are enzymatically active, indicates that entry involves something more specific than simply passive inclusion of toxin within the aqueous phase of pinocytotic vesicles. Furthermore, evidence from the study of CRM 197 strongly suggests that the initial interaction is with specific receptors on the cell surface. This CRM (87, 160, 162) which contains an inactive fragment A and a functional fragment B, has the interesting property of *inhibiting the action of toxin on protein synthesis in tissue culture*, although it does not inhibit ADP-ribosylation activity in cell-free systems (Fig. 15). The effect has been studied in some detail, and it has been concluded that the CRM acts in a strictly competitive manner with a K_D of about 10^{-8} M.

The competitive effect of CRM 197 has also been demonstrated qualitatively in whole animals using the skin reaction as an assay for toxin action (87), but diffusion of the competitor from the area of injection prevents precise quantification. Competition in lethality tests is not feasible to show because of the large amount

of material required to achieve an effective molar concentration of competitor in a whole animal.

The simplest explanation of these results is that CRM 197 and toxin bind competitively to specific receptors on the surfaces of cells. The interaction presumably involves the B portions of the molecules, and thus the binding affinities of both should be the same. Presumably whatever is attached to the receptors is brought within cells at a certain rate. Entry of the CRM alone, with its inactive fragment A, has no effect on protein synthesis, but blockage of toxin attachment and entry would have a protective effect.

There is little information relevant to the nature, number, or distribution of the putative toxin receptors. There are contradictory reports regarding whether the receptors are sensitive to

various proteases (48, 104a). Neuraminidase, lysozyme, and hyaluronidase seem not to affect the intoxication process (48). It was estimated from studies of binding of ^{125}I -labeled toxin that a HeLa cell can bind less than 50 molecules of toxin (125). However, the number may be much greater because of release of absorbed toxin during washings to remove unabsorbed material. In fact, the label found associated with the cells may have represented toxin in the process of entry or already within cells.

Entry and Activation Processes

These steps represent the major gaps in our knowledge of how the toxin affects cells. The reversibly bound toxin, or at least the active fragment, must traverse the plasma membrane either by an active mechanism, or by dissolution in the membrane. If an active mechanism is operative the toxin may arrive in the cell interior within a pinocytotic or phagocytic vesicle, which might fuse with a lysosome prior to release of its contents. Lysosomal proteases may therefore perform the essential nicking of intact toxin. Presumably at least the protease-resistant fragment A portion would survive this digestion process and be released into the cytosol proper, where it could inactivate EF-2. Glutathione may promote the terminal reductive activation of the toxin. This compound is present at concentrations (1 to 6 mM) which are sufficient to reduce nicked toxin rather rapidly.

Alternatively, if passive dissolution of toxin in the plasma membrane is involved, the hydrophobic fragment B portion may act as a carrier through the lipid phase. One can envision that the hydrophilic A fragment might be released into the cytosol, leaving the B portion attached to the membrane. Nicking might occur within the membrane or at the exterior or interior surface and might perhaps be catalyzed by proteases involved in protein turnover. Such questions are likely to remain speculative until attachment of very small amounts of toxin can be measured directly and the fate of the attached molecules can be followed.

At the present time we know of certain factors which apparently affect the intoxication process at the level of entry, but the interpretations of their effects is open to question. For example, at 10 C or lower the toxin is able to adsorb, but is maintained in an antitoxin-sensitive state, presumably at the cell surface (95, 150). Ammonium salts, certain aliphatic amines, and NaF also seem to inhibit entry of the toxin without preventing adsorption (47, 48, 94, 95), and homologous interferon partially

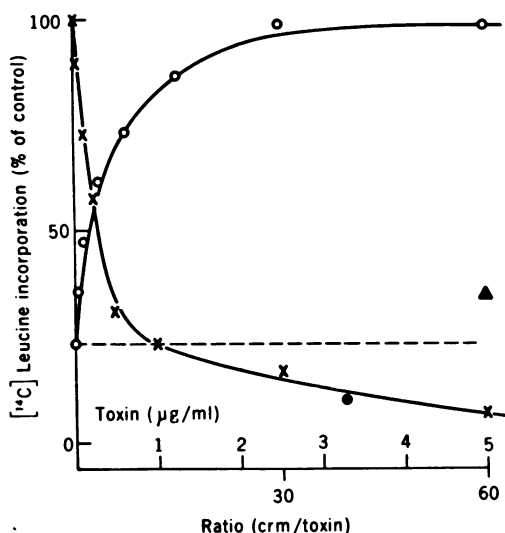


FIG. 15. Competition between CRM 197 and toxin for HeLa cell binding sites. Samples of cells in roller tubes were incubated with the concentration of toxin indicated for 3.5 h, and [^{14}C]leucine was then added. After an additional 3.5 h, the cells were harvested and the incorporated leucine was measured (\times). The curve formed by the open circles represents the incorporation observed when the cells were treated with a constant concentration of toxin (1 $\mu\text{g/ml}$) plus a variable concentration of CRM 197. The ratio of the CRM to toxin is indicated below the horizontal axis. Incorporation of leucine in the presence of toxin alone at 1 μg per ml is indicated by the dashed line. The closed circle and triangle show leucine uptake in the presence of CRM 45 and purified toxoid in ratios to toxin of 40:1 and 60:1, respectively. Reproduced from reference 160 by permission of the author and publisher (copyright 1972 by the American Association for the Advancement of Science).

protects against the toxin, perhaps by an effect at the same level (110). In contrast, the sensitivity of toxin-resistant mouse cell lines to the toxin may be enhanced by polycations, such as polyornithine or poly-DEAE-dextran (106). The effects of these factors may perhaps be explained by their stimulation or inhibition of endocytotic activity, but further studies are needed to confirm this. If this is the case, the toxin would presumably enter the cell attached to membrane receptor sites on the interior surfaces of endocytotic vesicles.

Cell lines which are resistant to the toxin will certainly be useful in studying the adsorption and entry processes. A block at this level has been inferred not only for cell lines from toxin-resistant animals, but also for toxin-resistant variants of normally sensitive cell lines. Moehring and Moehring have selected for clones of human KB cells which are resistant to moderate levels of toxin (107). The change to toxin resistance seems to be stable, in that resistance is maintained when the cells are cultivated in the absence of toxin. Protein synthesis was studied in extracts of one of the resistant strains and was found to remain highly sensitive to the toxin, thus indicating that resistance was not due to an alteration of EF-2. Other evidence suggested that changes had occurred in the cell surface. Thus, resistant clones formed colonies with different morphology and were resistant to dispersion by trypsin. In addition, toxin resistance seemed to be accompanied by an increased resistance to certain RNA viruses, including poliovirus, Mengo virus, vesicular stomatitis virus, and Newcastle disease virus (108). Adsorption of the viruses occurred, but uncoating and release of viral RNA seemed to be inhibited in certain resistant cells. In two strains there was also a reduced production of viral mRNA. It has been suggested that an alteration may have occurred in a proteolytic activity required both for nicking of toxin and uncoating of certain viruses (108), but the relationship of virus resistance to toxin resistance remains uncertain.

Kinetics of Entry and Turnover

By whatever mechanism toxin enters, it begins to inactivate EF-2 within minutes, long before the end of the lag period. Gill et al. have shown that the total elongation factor activity in the supernatant fraction of toxin-treated cells drops markedly within 20 min although protein synthesis is not affected until 1 h (60). The discrepancy here apparently results from the fact that EF-2 is not normally the limiting

factor in protein synthesis. Gill and Dinius have shown that EF-2 is present in a molar ratio of 1.2 molecules per ribosome in a variety of tissues (57). Apparently only after this ratio drops as a result of ADP-ribosylation to some as-yet-undetermined low value does EF-2 begin to limit the rate of protein synthesis.

Entry of toxin continues well beyond the end of the lag period, but the exact duration is not known. Protracted entry is well demonstrated with CRM 176, which has a fragment A with 8 to 10% the normal specific activity, and which inhibits protein synthesis in HeLa cells to a lesser extent than similar levels of toxin (162). If cells are treated with CRM 176 for 1.5 or even 3 h, and then washed, the decline in the rate of protein synthesis halts, and the cells recover. Inasmuch as the decline continues in cells further treated with the CRM, entry must be occurring at these times. In cells treated with toxin for 1.5 h and resuspended in toxin-free medium (containing CRM 197 to prevent further entry of toxin), the decline in the rate of protein synthesis was only marginally affected by the removal of toxin, and the cells did not recover.

The recovery of CRM 176-treated cells presumably depends on degradation of the A176 present in the cytoplasm, and it is reasonable that any protein brought within cells should be subject to the protein turnover system (66). The greater stability and/or specific activity of normal fragment A presumably accounts for the lack of recovery of cells treated similarly with toxin.

After the lag period the decline in the rate of protein synthesis in toxin-treated cells appears to follow first-order kinetics (162). It has been suggested that this implies the presence of a virtually constant concentration of fragment A within cells, presumably a steady state between entry and degradation. This is plausible, but other explanations for such kinetics must be considered in view of the number of unknowns affecting the intracellular reaction.

Calculations of the specific activity of fragment A in the intracellular milieu cannot yet be performed with confidence because of the large number of uncertainties in variables affecting enzymatic activity and the substrate activity and availability of EF-2. Therefore, it is not possible to predict accurately on this basis the concentration of intracellular fragment A needed to inhibit protein synthesis at a given rate. Unfortunately this concentration is too low to measure directly.

There has been considerable speculation

about the minimum number of toxin molecules required to kill a cell (48, 61, 122, 123). With certain cell lines it is clear that concentrations of toxin equivalent to several hundred molecules per cell are sufficient to cause death within 3 to 4 days (54, 130). The actual number may be much lower, however, because a significant percentage of the toxin may not have been taken up by cells. Studies of the uptake of ^{125}I -labeled toxin by HeLa cells suggest that 50 molecules of toxin or less may be sufficient to cause cell death (122), and from the fact that the kinetics of adsorption of a lethal dose of toxin by HeLa cells are first order, it has been suggested that a single molecule of toxin may be lethal (48). In view of the complexities and unknowns in the intoxication process, one would like to have independent confirmation of the last figure.

IMMUNOLOGY

Avidity Correlates with Antibody Against Fragment B

Only recent studies pertaining directly to the structure and activity of the toxin will be considered here; earlier studies which contributed so much to the understanding of antigen-antibody interactions (121) will be ignored.

Pappenheimer, Uchida, and Harper have reported an immunological study of the toxin molecule making use of the fragmentation techniques and CRMs available (127). As studied by immunodiffusion in the presence of 0.5 M urea, fragments A and B are immunologically distinct, and both formed lines of partial identity with toxin when tested against antitoxin (Fig. 16) (9, 127). CRM 197 is immunologically indistinguishable from toxin, whereas CRM 45 shows partial identity. Both fragments A and B form lines of partial identity with CRM 45.

Other findings on antisera or absorbed sera directed against specific portions of the toxin molecule were less predictable. By adsorption of antitoxin with CRM 45, a serum was obtained which was specific for the C-terminal 17,000 daltons of the toxin. Interestingly, the *avidity* of this serum was greater than the unadsorbed sample; that is, it had an increased capacity per unit of antibody protein, for *neutralization* of toxicity. This finding suggested that antibodies against the C-terminal region of the toxin have a greater capacity for neutralization than those against the remaining portion.

Other results neatly confirmed this prediction. Thus antisera against purified fragment A, and which quantitatively precipitated the frag-

ment, were found to contain almost no capacity to neutralize toxicity. Furthermore, among a number of antisera tested, the avidity was inversely related to the proportion of anti-fragment A, expressed as percent of total toxin-precipitable antibody. Thus, there appears to be a basic difference in neutralizing capacity of antibodies directed against the N- and C-terminal regions of the molecule.

That antibodies against a region necessary for attachment to cells should block toxic activity is not surprising, but the lack of neutralizing capacity of those against the fragment A region requires explanation.

The low avidity of anti-fragment A antibodies could be due to a low affinity constant, permitting dissociation of toxin-antitoxin complexes *in vivo*. Alternatively, an antibody of high affinity might become detached during or after the adsorption and entry of toxin into cells. For example, this might occur as a result of an altered conformation of the receptor-bound toxin, or might take place after traversal of the plasma membrane, perhaps following cleavage or reduction events. Complexes of antitoxin with fragment A or nicked toxin are dissociated in the presence of thiols, yielding active fragment A (127). (This finding probably explains the early observation that washed toxin-antitoxin floccules are slightly active in inhibiting protein synthesis in cell-free systems [35, 123]).

A more complex situation is suggested by the fact that anti-fragment A antibody precipitates only a fraction of ^{125}I -labeled toxin (127). Furthermore, in antibody excess, unprecipitated toxin did not appear to be complexed with immunoglobulin, as judged by Sephadex chromatography. This suggests that the fragment A determinants may be obscured in a fraction of the population of toxin molecules. This would partially explain the lack of neutralizing capacity of anti-fragment A antibodies.

Immunogenicity and the Mechanism of Toxoiding

Pappenheimer et al. reported other interesting results relevant to the *immunogenicity* of diphtheria toxin (127). Antitoxins elicited against formaldehyde-treated toxin, or toxoid, in rabbits or horses varied in content of anti-fragment A antibodies and hence in avidity. In two sera of moderate to high avidity, about 30% of the toxin-precipitable antibodies were against fragment A, whereas another horse-antitoxic globulin preparation (number 5353) of particularly high avidity was found to contain

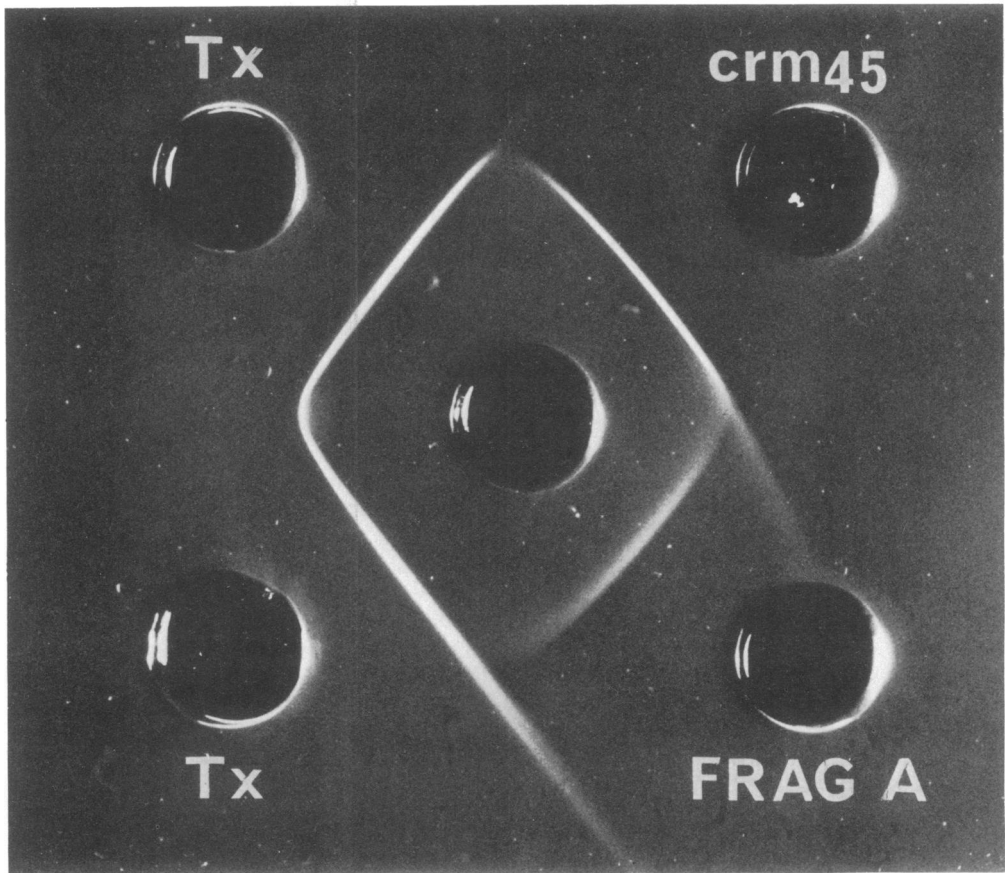


FIG. 16. Immunodiffusion against horse antitoxin in agar containing 0.5 M urea in buffered saline. From reference 127 by permission of the author and publisher.

only traces of anti-fragment A. In addition, we have found another horse-antitoxin with unknown avidity in which precipitation of toxin may be quantitatively inhibited by fragment A (J. Kandel and R. J. Collier, unpublished data). Human antisera to the toxin have been found frequently to contain substantial quantities of anti-fragment A (9).

Experiments on the immunogenicity of CRM 197 are interesting with respect to these variations. Antisera against CRM 197, like those against CRM 45 or free fragment A, contained a high proportion of anti-fragment A antibodies, despite the fact that CRM 197 has a normal fragment B and is immunologically indistinguishable from toxin. However, formaldehyde-treated CRM 197 produced sera of high avidity with little or no anti-fragment A.

These results may perhaps be explained by a protective effect of formaldehyde on the sensitivity of CRM 197 or toxin against proteolytic action in the body. The fragment B region of

toxin is relatively sensitive to proteolytic attack, and after injection of toxin this region may be attacked rapidly, leaving only the protease-resistant fragment A to elicit antibody formation. Presumably, formaldehyde protects the B region and hence preserves its immunogenicity.

Of course formaldehyde also serves to detoxify the toxin. By what mechanism does detoxification occur? Formaldehyde reacts primarily with the ϵ -amino groups of lysine residues, but may also form methylene bridges between the lysines and tyrosine or histidine (16). Inactivation of toxin might occur simply as a result of blockage of a critical group or groups of fragment A or fragment B, or alternatively may result from cross-linking of the two fragments, thus preventing dissociation and the expression of enzymatic activity.

The available evidence indicates that toxoid lacks receptor-blocking activity and contains only a trace, if any, ADP-ribosylation activity. Also, there is evidence that nicked toxin is

resistant to dissociation by thiols and SDS after formaldehyde treatment (9, 100, 104). It is clear, then, that the fragment B region is inactivated, but it is not yet known whether the lack of activity of fragment A within toxoid is due to modification of essential groups or to the inability to be dissociated from B. Beugnier and Zanen have reported evidence favoring the former possibility (13). All of the mechanisms mentioned may in fact occur and contribute to various degrees to detoxification.

BIOLOGICAL FUNCTION AND ORIGIN OF DIPHTHERIA TOXIN

With some solid information on the structure and biochemical activity of diphtheria toxin in hand, one must consider whether we are any closer to understanding the origin and *raison d'être* of the toxin. How did the toxin evolve, and what selective advantage accrues to phage carrying the *tox*⁺ gene and/or to their host bacteria?

Biological Function

In essence, we must examine the possible role(s) of the toxin in the lysogen-human host interaction, and that between the phage and its bacterial host.

(i) **The lysogen-human host interaction.** Our tendency in thinking about a selective advantage or biological function of toxin production is to focus on the dramatic, highly malignant infections of *C. diphtheriae*, but in so doing we are probably misled. In early surveys of naturally acquired immunity to the toxin in children, it was found that most infections sufficient to cause such immunity do not result in serious disease (25). Thus, as with many other pathogens, the number of subclinical infections appears to outnumber greatly the clinical ones. In addition, it seems quite certain that death of the human host does not benefit either the lysogen or the phage, inasmuch as a deceased host is unable to transmit either. Both of these considerations lead us to conclude that the selective advantage of toxicity, if any, is probably exerted in mild or symptom-free upper respiratory infections, rather than the severe forms of the disease. Thus, highly malignant diphtheria may perhaps best be viewed as a biological accident, of little significance to the long-term survival of toxigenic strains.

We certainly do not know the true function of the toxin, but one can conceive that irritation of the epithelial tissues of the upper respiratory tract by the toxin might facilitate growth, or perhaps promote dissemination by inducing

coughing or sneezing. Alternatively, the toxin might provide a local defense against phagocytosis. Whatever the effect, it is somewhat anticlimactic in comparison with the more malignant manifestations of toxicity.

(ii) **The phage-bacterium interaction.** This problem is at least amenable to experimental study. Given that the structural gene for toxin resides within a phage genome, one must first ask whether the toxin plays a direct role in phage replication. It has been argued that the toxin cannot be essential for phage replication inasmuch as the mutations producing CRMs 30, 45, 176, 197, and 228 do not affect the one-step growth curves after ultraviolet induction of lysogens carrying these mutants (158, 159). However, mutations which alter the toxicity of the toxin would not necessarily block a different function in phage replication. Moreover, it should be noted that the isolation procedure employed selected for viable phage mutants in the first place, thereby inserting a bias into the results.

Iglewski and co-workers have published evidence suggesting that fragment B from the toxin may form part of the phage B virion (51).

Virions of a virulent mutant of β , purified by differential centrifugation and isopycnic banding in CsCl gradients, show a major protein band in SDS gels which comigrates with fragment B. Also, it was reported that such purified phage exhibited blocking activity in protecting KB cells from diphtheria toxin, implying the presence of functionally active fragment B. A major weakness in these results is the possibility of copurification or adsorption of fragment B which is not an integral part of the phage. Also, one would like more positive evidence (e.g., amino acid sequence data) confirming that the protein observed on gels is in fact fragment B.

At present the data seem insufficient to decide definitely for or against an essential function for the toxin in phage replication. Even if no essential role can be found, it is still possible that the toxin could perform a direct but nonessential function in replication. Finally, in the absence of the latter, the phage might benefit indirectly by an unknown mechanism by virtue of the effects of toxin on the human host.

Origin

Given the location of the structural gene for diphtheria toxin, it is simplest to suppose that *tox*⁺ originated from a phage gene, even if it now serves neither an essential nor a facultative function in phage replication. This notion has

recently received indirect support from a finding by Goff in *E. coli* infected with phage T₄ (64, 65). In such cells the α subunit of RNA polymerase was found to be modified by covalent attachment of the ADPR moiety of NAD⁺.

The facts that this modification (i) involves the same group-transfer from the same pyridine nucleotide substrate, (ii) is catalyzed by a phage-encoded enzyme, and (iii) is the first example in prokaryotes of ADP-ribosylation of a protein, invite speculation that it may be related to the origin of diphtheria toxin. One can conceive that the ADP-ribosylation activity of the fragment A portion of the toxin may have originated from a phage enzyme which catalyzed a similar group-transfer to a subunit of RNA polymerase or some other protein involved in phage replication. The gene for this enzyme might have been joined by gene fusion with that of another protein (the phage coat protein?) corresponding to the B portion of the toxin, with gene duplication accounting for essential gene functions destroyed in the fusion process.

Whether this hypothesis deserves much credence will depend in part upon whether ADP-ribosylation of proteins is found in other phage infections, including those by corynephages. No significant incorporation of ADPR from NAD⁺ has been detected in extracts from the PW-8 strain of *C. diphtheriae* incubated either in the presence or absence of fragment A, suggesting that such cells contain no protein acceptor for ADPR. However, such an acceptor might easily have been overlooked if it were labile or present in low concentrations. Goff has found (personal communication) that fragment A does not catalyze ADP-ribosylation of the α subunit of *E. coli* RNA polymerase, but the corresponding enzyme from diphtheria bacilli has not been tested.

Alternatively one can conceive that the toxin may have originated from NAD⁺-glycohydrolase or a NAD⁺-linked dehydrogenase. The transition to an enzyme capable of transferring the ADPR moiety to a specific protein might well be relatively minor in either case. Presumably the substrate activity of EF-2 could only be accounted for by a chance affinity for the enzyme.

Other authors have suggested that the gene for diphtheria toxin may have originated in the genome of a eukaryotic host (158). The apparent rationale for this is that the only known efficient protein substrate for the toxin is a eukaryotic intracellular protein. It is supposed that the ancestral gene for the toxin might have encoded a regulatory protein which modified

the activity of EF-2 or a similar protein, and that the phage picked up this gene through its close association with a eukaryotic host. There is, however, no evidence for such a regulatory protein at the present time.

None of the above theories offers an explanation for the origin of the binding site on toxin for specific cell surface receptors. Speculation on this point is largely futile until we know more about the chemistry of the receptors.

SUMMARY AND CONCLUDING REMARKS

The following is suggested as a possible sequence of events in the action of diphtheria toxin in clinical diphtheria.

(i) The toxin is excreted by *C. diphtheriae* in the form of a single polypeptide chain, molecular weight 63,000, and gains entry into the body through lesions in the epithelium at the site of the infection.

(ii) It is transported throughout the body by the blood and lymphatic systems and attacks many or most types of cells to which it is exposed.

(iii) The toxin attaches with a K_D of about 10 nM to specific receptors on the cell surface, the nature and number of which are unknown.

(iv) While attached to the surface membrane, it is transported within the cell by endocytosis. The toxin may have been partially nicked before it arrives at the cell surface, but nicking may also occur intracellularly, perhaps within the endocytotic vesicle through the action of lysosomal or other proteases.

(v) A certain percentage of the toxin molecules, or at least the fragment A portion, survives and is released into the cytosol, where reduction and release of fragment A may be promoted by glutathione.

(vi) Fragment A inactivates the free form of EF-2 by catalyzing attachment of the ADPR moiety of intracellular NAD⁺. The modified factor can still attach to ribosomes and bind GTP, but is inactive in promoting translocation.

(vii) EF-2 apparently does not normally limit the rate of protein synthesis, and only after a sizable fraction has been inactivated does the rate of protein synthesis decline. After this inhibition, other cellular processes are affected, producing necrosis, gross physiological changes, and sometimes death.

Certain aspects of this sequence are well supported by data, in particular, the enzymology of the toxin and its effect on protein synthesis. On the other hand, we are badly in

need of more evidence regarding the attachment and entry phenomena. In view of the rapid advance of techniques for studying biological membranes, one can hope that such data will be forthcoming soon.

In conclusion one should perhaps consider possible implications of the studies reviewed here. First, what generalizations are possible?

With regard to the ADP-ribosylation reaction, there is no evidence to suggest it may be in any way general among toxins (163). Although the precise actions of most bacterial toxins are not known, their gross activities give no suggestion that they may act like diphtheria toxin.

On the other hand, the structure-activity relationships evidenced in diphtheria toxin may represent a more general phenomenon. In certain other bacterial toxins and in two toxins from plants there is evidence that the functions of attachment to cells and of toxic modification of cells (the effector function) may reside in different polypeptide chains. The data are clear in the case of the plant toxins, ricin and abrin, both of which inhibit protein synthesis by mechanisms unrelated to ADP-ribosylation (117-119). Each of these contains two chains in disulfide linkage. One of the chains (the B chain) is active in attachment to cells, and the other (A chain) inhibits protein synthesis. The activity of the A chain is apparently expressed only after reduction and dissociation from the B chain. Both toxins attach to galactose-containing receptors on the cell surface.

Another example is cholera toxin. The toxin appears to be dissociable under nonreducing conditions into a subunit (molecular weight 66,000) which is devoid of biological activity but which competitively binds to the same membrane site as the toxin, and another (molecular weight 36,000) which presumably has the effector activity of the toxin (43). The toxin appears to act by stimulating adenyl-cyclase in cells by an undefined mechanism. The binding sites on the cell surface have been shown to be gangliosides (41, 42).

Finally, in some clostridial toxins there is evidence for a form containing two disulfide-linked chains. In certain botulinum toxins there is an activated form created by proteolytic nicking, and reduction produces a loss of toxicity (10, 44, 152). A similar situation may exist with tetanus toxin (39). The specific functions of the chains are not well defined in either case.

It will be interesting to know whether the receptor-attachment and effector functions of protein toxins generally reside on separate peptide chains. The various toxins almost certainly

do not have a common origin, but evolutionary constraints may exist which dictate such a structure. Different locations of the receptor-binding and effector activities might be favored if the receptor-attachment region remains permanently fixed to the membrane; release of the effector region into the cytosol might be facilitated if it were on a separate chain.

Diphtheria toxin has also proven to be a useful tool in studying certain aspects of protein synthesis. In addition to inactivating EF-2 and blocking translocation specifically, the toxin offers the advantage of attaching a specific radioactive label to EF-2. This has proven useful in quantifying EF-2 in crude tissue extracts and in studying the mechanism of translocation in detail (11, 12, 36, 57, 59, 153-155). An example of the latter application is recent work using ADPR-EF-2 to identify by chemical cross-linking the proteins to which EF-2 binds on the ribosome (157).

What about possible medical applications of the research? It was hoped that a means of therapy useful in clinical diphtheria beyond rescue with antitoxin would have been revealed. Knowledge of the intracellular events might have provided a means of reversing or at least limiting the lethal action. Unfortunately, this has not occurred. To reverse the reaction with nicotinamide, one would need intracellular levels of this compound which themselves would be toxic even if they were attainable. Although certain of the CRMs block the toxin's activity on whole cells, they are no more useful than antitoxin because they do not affect toxin which has already entered the cell.

With regard to prophylaxis against diphtheria, there is really no need to improve upon the efficacy of toxoid for immunization. However, use of one of the nontoxic CRMs for the preparation of toxoid should eliminate the occasional problem of partial reversion of toxoid to the toxic state (1).

Finally, there appears to be a remote possibility that our knowledge about diphtheria toxin may have practical applications outside the realm of bacteriology. It has been reported recently that certain types of malignant cells from mice or humans may be inherently more sensitive to diphtheria toxin than normal cells from the corresponding organism (26, 86). In addition, there have been other reports suggesting that the toxin may be converted into a more specific cytotoxic agent by covalently conjugating it to antibodies directed against specific cellular antigens (114, 128, 143). It is difficult to predict if such studies may ever reach the stage

of practical therapy, but this possibility should not be neglected.

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